

A thymic stromal lymphopoietin polymorphism may provide protection from asthma by altering gene expression

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ABSTRACT

Background: Genome-wide association studies have identified associations of the single nucleotide polymorphism rs1837253 in the thymic stromal lymphopoietin (*TSLP*) gene with asthma, allergic disease and eosinophilia. The *TSLP* gene encodes two isoforms, long and short, and previous studies have indicated functional differences between these two isoforms.

Objective: We investigated the expression of these TSLP isoforms in response to a pro-inflammatory signal, and the role of the rs1837253 genotype in gene isoform regulation.

Methods: We cultured nasal epithelial cells of asthmatic and non-asthmatic subjects and evaluated poly(I:C)-induced TSLP protein secretion using multiplex protein assays and gene expression profiles of the TSLP isoforms, and related genes, using real-time qPCR. We correlated these profiles with rs1837253 genotype.

Results: Asthmatic nasal epithelial cells exhibited increased TSLP protein secretion compared to nasal epithelial cells from healthy controls. The long TSLP isoform was more responsive to poly(I:C) stimulation. Additionally, the minor T allele of rs1837253 was less inducible than the major C allele, suggesting differential regulation; this may explain the “protective” effects of the T allele in asthma.

Conclusion: Our results provide important insights into the differential regulation and function of TSLP isoforms, including the role of *TSLP* rs1837253 polymorphisms in allergic inflammatory processes.

Clinical Relevance: The key finding on the influence of TSLP genetic variation on disease expression/endotype could provide basis for investigation into targeted biologics for anti-TSLP therapies.

INTRODUCTION

Thymic stromal lymphopoietin (TSLP) is an important regulator of inflammatory responses in an array of allergic diseases. TSLP is produced by multiple cells, including epithelial cells, basophils, and mast cells, and drives inflammatory responses through dendritic cells[1], mast cells, and CD34+ progenitor cells[2]. In bronchial epithelial cells (BECs) of asthmatic subjects, there is increased *TSLP* mRNA expression in comparison to healthy controls; this

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differential expression is associated with the degree of airway obstruction[3]. Higher gene and protein expression of *TSLP* are also observed in bronchoalveolar lavage from asthmatics and chronic obstructive pulmonary disease (COPD) patients[3–5].

Respiratory tract infections in early life are associated with later development of asthma[6]. Viral infection and replication in the airway epithelium trigger the release of pro-inflammatory mediators, including *TSLP*, to initiate the inflammatory cascade[7]. The expression of *TSLP* in human airway epithelial cells is inducible by rhinoviruses[8], through an infection that may increase an individual's risk for asthma[9]. Polyinosinic:polycytidylic acid (poly(I:C)), a form of double-stranded (ds) RNA, can be utilised as a synthetic toll-like receptor-3 (TLR3) agonist, mimicking the natural TLR3 ligand, viral dsRNA. Poly(I:C) has indeed been shown to be a potent inducer of *TSLP* expression[8,10–12].

A single nucleotide polymorphism (SNP) rs1837253 in the upstream region of *TSLP* has been shown to be highly associated with asthma [13,14]; multiple genome-wide association studies (GWAS), including meta-analyses spanning several ethnic groups[15–19], have confirmed and extended these associations to several asthma phenotypes (such as the eosinophilic endotype). The rs1837253 T allele (minor allele) is associated with a lower risk of asthma and hence considered to have a “protective” effect on asthma development[13,20,21]. The *TSLP* gene encodes two transcript variants in humans: a long *TSLP* isoform (lTSLP), and a short *TSLP* isoform (sTSLP)[22]. The C terminal of the long isoform, common to both isoforms², can be used to evaluate total *TSLP* transcripts. The two isoforms seem to play differential roles in inflammatory responses: pro-inflammatory signals induce expression of the long isoform, which is associated with allergic inflammation, while the short isoform is expressed constitutively and may have anti-inflammatory or anti-microbial effects[23–25].

In this study, we investigated the roles of the short and long isoforms of *TSLP* in response to an inflammatory signal. We examined the effects of the *TSLP* SNP rs1837253 genotype on *TSLP* gene and protein expression from NECs following stimulation with poly(I:C), as well as expression differences between asthmatic subjects and controls. We aimed to identify functional differences associated with SNP genotypes to suggest a possible mechanism by which the rs1837253 genotype may exert its “protective” effect.

METHODS

Study Subjects

This study was approved by the Hamilton Integrated Research Ethics Board (REB Project #13-479) and all subjects provided written informed consent. Eligible subjects were non-smokers, aged 18 to 65. All participants had mouthwash samples collected and skin prick tests (SPT) performed. Atopy was defined as at least one positive SPT (> 2 -mm wheal above a negative control) to one of 14 common aeroallergens. Asthma was defined in subjects based on the results of methacholine challenge testing if the concentration of inhaled methacholine that provoked a 20% decrease in the forced expiratory volume in 1 second (PC20) was ≤ 16 mg/mL. No subjects required medication for treatment of asthma or allergies. All asthmatic subjects with allergy were atopic with mild asthma, which was defined as requiring only SABA as needed; at the time of sampling, asthma stability was confirmed by SABA use less than twice during the last 7 days. Subjects were excluded if they had had asthmatic exacerbations or respiratory tract infections within 4 weeks of the study start date, or other underlying diseases. Subjects treated with nasal steroids, antihistamines and anti-leukotrienes unable to discontinue these medications for 2 weeks before collection of nasal epithelial cells were also excluded.

Isolation of genomic DNA and genotyping

DNA was extracted from mouthwash samples using QIAamp Blood Mini Kits (Qiagen, Toronto, ON, Canada) according to manufacturer's supplementary protocol for mouthwash samples. Genotyping was then performed using Taqman genotyping assay for rs1837253 (Assay C__11910823_20, Life Technologies, Burlington, ON, Canada). The genotyping data were tested for Hardy-Weinberg equilibrium using the χ^2 test.

Cell culture, qRT-PCR, and Protein Measurements

Detailed methods are outlined in the supplementary information. Briefly, primary nasal epithelial cells (NECs) were derived from the inferior nasal turbinate using Rhino-Probe nasal curettes (Arlington Scientific, Springville, UT). NECs were expanded and cultured as previously

described by Muller *et al.*[26] with some modifications. Poly(I:C) stimulation was performed as previously described by Hui *et al.*[20]. RNA was extracted from the NECs using RNeasy Mini-kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The samples were reverse transcribed using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to manufacturer's instructions and complementary DNA samples were aliquoted and stored at -80°C. *TSLP* gene expression was assessed using SsoAdvanced™ Universal® SYBR Green Supermix (Bio-Rad, Hercules, CA) with a MX4000 Stratagene detection system according to the manufacturer's instructions. Protein levels of TSLP, GM-CSF, IFN γ , TNF- α , IL-8, IL-33 and IL-5 from the cell culture supernatants following 24h poly(I:C) treatment were assessed using Bio-Plex assays (Bio-Rad, Hercules, CA), according to manufacturer's recommendation.

Reporter Gene Assay

DNA fragments containing *TSLP* rs1837253 alleles were inserted into a pGL4.23[luc2/minP] vector using restriction enzyme sites. Plasmid-carrying *E.coli* bacterial colonies were grown and plasmid DNA extracted, which was used to transfect A549 cells. Poly(I:C) treatments were added 24-hour post-transfection. Dual-Glo® Luciferase assay system (Promega) reagents were used to measure Luciferase reporter gene activity. See supplementary information for methodological details.

Statistical Analysis

All data are expressed as the mean $\pm$ SEM. Alpha level of significance was set at 0.05. Data analysis was completed using GraphPad Prism 5.0 (GraphPad Software, LaJolla, CA, USA) software or R statistical programming to perform students t-test and ANOVAs. Post-hoc analyses for between-group comparisons were performed using Tukey's multiple comparisons test for one-way ANOVA and Bonferroni's test for two-way ANOVAs.

RESULTS

Subject Characteristics

From December 2015 to June 2017, 45 subjects were enrolled in the study. Of these subjects, 15 were asthmatics and 30 non-asthmatics. Of the 15 asthmatics, 14 were atopic. Of the 30 non-asthmatic subjects, 23 were atopic. The *TSLP* rs1837253 genotype distribution was as follows: 8 subjects homozygous for the minor allele (TT), 19 subjects homozygous for the major allele (CC) and 18 heterozygous subjects (CT) (Table 1).

Nasal epithelial cell TSLP protein expression is higher in asthmatics and in subjects homozygous for the major C allele

NEC cultures were stimulated with 25 µg/mL poly(I:C) for 24 h and the supernatant was analyzed for protein levels using a multiplex cytokine and chemokine assay. Total TSLP protein was significantly elevated in asthmatic subjects compared with non-asthmatics (Figure 1). Additionally, there were significantly higher TSLP protein levels in subjects homozygous for the major allele (CC) compared to those homozygous for the minor allele (TT). No significant differences were found in the protein expression of IL-33, IL-8, IL-5, GM-CSF, IFN γ or TNF α (Figure 2). TSLP protein expression was also stratified by both genotype and asthmatic status (Supplementary Figure 1).

Poly(I:C) stimulates *TSLP* gene expression in a time-dependent manner

Nasal epithelial cells were stimulated with poly(I:C) (25 µg/mL) for 0, 3, and 6 hours. The gene expression was measured using real-time quantitative PCR to determine the expression fold change in the stimulated cells versus unstimulated control cells. The expression of total TSLP transcripts was elevated in a time-dependent manner at 3h ($p < 0.01$) and 6h ($p < 0.001$), compared to the unstimulated cells (Figure 3A). The long isoform was significantly increased at both 3 and 6 h, whereas the short isoform was found to be increased at 6h (Figure 3B, 3C).

TSLP gene expression is altered following poly(I:C) stimulation

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NEC mRNA expression was analyzed in relation to genotypes. When comparing the gene expression across genotypes, total TSLP was significantly increased at the 6h time point across genotypes, where C allele carriers (CC + CT) exhibited higher expression in comparison to the TT homozygous subjects ($p=0.003$) (Figure 4A). When the expression was further stratified by isoform, the long isoform was elevated after 6h poly(I:C) stimulation for C allele carriers, compared to TT homozygous subjects (Figure 4B). The short isoform was not differentially expressed across genotypes (Figure 4C).

When comparing the gene expression of *TSLP* (total, long and short isoforms) in atopic and non-atopic individuals, regardless of asthmatic status, there were no associations between atopy *per se* and gene expression of these regulatory proteins in NECs.

When stratified by rs1837253 allele (C or T), total expression of poly(I:C)-induced TSLP was higher in asthmatics compared to non-asthmatic individuals (Figure 5A). However, the short and long isoforms separately showed no significant differences between allelic groups at 6h (asthma and no asthma) (Figure 5B, 5C).

Luciferase reporter gene assays results

Reporter gene assays were performed using A549 cells transfected with plasmids of each allele (C and T). Luciferase activity from untreated cells was significantly higher ($p=3.6 \times 10^{-4}$) in the cells containing the rs1837253-T allele compared to the C allele (Figure 6). After poly(I:C) treatment, luciferase activity significantly increased in cells transfected with the C allele plasmid, but not the T allele plasmid. The increase was in a dose-dependent manner for the C allele (Figure 7) and was absent in cells transfected with the T allele. Six replicates were used for each treatment dose for each allele and all data points measured were plotted i.e. no outlier identification or removal were performed. Given the use of the proper controls (no treatment, empty vector control and Renilla transfection control) and the plating of both alleles in the same culture plates, we speculate the variability observed for one allele over the other is biological and not due to technical bias.

DISCUSSION

Viral infections in early life are associated with the development of asthma and allergic disease later in life [6], and many asthma exacerbations are preceded by viral infections[27,28]. Viral infection induces epithelial cells to secrete high levels of TSLP. In this study nasal epithelial cells were used as they are an excellent model for lower airway epithelial cells, and provide a much less invasive solution to investigation of airway diseases[29–31]. Consistent with previous studies on small airways epithelial cells, NECs, and BECs[8,10–12], our results indicate that *ex vivo* exposure to a dsRNA analogue, poly(I:C), induces a time-dependent expression of long and short TSLP mRNA isoforms. Although both the lftTSLP and sftTSLP were upregulated following stimulation with poly(I:C), the long isoform was much more responsive to stimulation, with fold-changes in gene expression of 3.9×10^5 and 2.1×10^6 after 3 and 6 hours, respectively. lftTSLP was upregulated in response to poly(I:C), in line with previous studies that have shown increased lftTSLP in response to allergic inflammatory triggers [23]. The high fold-change of lftTSLP is likely due to its very low expression levels at steady state, while the short form is expressed constitutively in much higher abundance. Our results further highlight a potentially important functional dichotomy between the two TSLP isoforms[22].

We also compared TSLP expression profiles by rs1837253 genotype, in response to poly(I:C) treatment. We showed increased levels of lftTSLP in subjects carrying the C allele. The subjects with no copies of the T allele (CC) had significantly higher expression of lftTSLP than subjects with one or two copies of the T allele (CT and TT). In a reporter gene assay, we observed the same direction of effect: treatment of A549 cells transfected with rs1837253 allele-specific plasmids with poly(I:C) led to significantly increased luciferase activity in a dose-dependent manner, *only* in the cells transfected with the C allele. Our observation that the cells containing plasmids with the T allele are not responsive to poly(I:C) stimulation suggests that there is a lower propensity for induction of the allergic inflammatory pathway in individuals carrying the T allele, revealing a possible mechanism for the “protective” effects noted in previous studies[24,25,32], and in alignment with our overall findings. The T allele may regulate lftTSLP expression to produce this “protective” effect by leading to reduction of the TSLP-

induced inflammatory response in individuals carrying that allele. *Our findings suggest that the SNP rs1837253 may induce differential responses to viral insult that contribute to the airway inflammatory phenotype through a specific isoform of the TSLP protein.*

A central question investigated in this study is the functional role of TSLP SNP rs1837253 in the asthmatic phenotype. We addressed this by comparing gene expression in asthmatics vs. controls, taking rs1837253 genotype into account. Due to the lower population prevalence of the minor allele T, we combined the heterozygous CT and homozygous TT minor populations into one group and compared gene expression in that group to the homozygous major allele group (CC). In the subjects carrying at least one T allele, *total TSLP gene expression was elevated in asthmatics compared to controls.* We also observed a trend for non-asthmatic subjects carrying the minor T allele to exhibit the lowest fold change in TSLP expression, suggesting that they have a decreased response to pro-inflammatory stimuli. This is a novel finding that connects a clinical phenotype (asthma) with SNP genotypes. When the lftTSLP and sfTSLP were analyzed individually, there were no significant differences found in their expression, although lftTSLP did exhibit a similar trend than the total expression of both isoforms together. A greater sample size is required to further clarify this result. These findings, combined with prior evidence, suggest a differential role of TSLP isoforms in asthma and its clinical expression. The increase in the total TSLP transcripts following poly(I:C) stimulation of respiratory epithelium and the differential expression of TSLP isoforms need to be further investigated, in more highly statistically-powered studies.

We also examined *ex vivo* NEC protein secretion of hematopoietic cytokines, TSLP, GM-CSF, IL-5, IL-8, IFN γ and TNF- α , to determine whether these NECs could be contributing directly to eosinophil/basophil differentiation or recruitment at different rates, according to genotype and asthmatic status. We found no differences in protein secretion of these key hematopoietic and regulatory molecules from NECs when comparing asthmatics to non-asthmatics and when comparing different genotypes of rs1837253. However, in our analysis of TSLP protein secretion, we found differences between the rs1837253 alleles, consistent with the mRNA findings. This indicates that the T allele is associated with decreased production of TSLP protein. Additionally, TSLP protein was decreased in non-asthmatic individuals compared to asthmatics,

reinforcing the existing literature about the involvement of TSLP in asthma development[20]. Our protein assay was not designed to detect the different isoforms of TSLP; validation at the protein level of our gene expression results, which showed differences between the short and long isoform of TSLP, is warranted.

A recent study has identified anti-TSLP therapy as a means to reduce airway bronchoconstriction and airway inflammation, especially airway eosinophilia, in patients with mild asthma[33]. Tezepelumab (AMG 157), a human anti-TSLP monoclonal immunoglobulin G2 λ that binds human TSLP and prevents receptor interaction, was able to reduce airway inflammation, blood and sputum eosinophils, and increase methacholine PC₂₀ in allergic asthmatics. Taken together with our current findings on differential responses according to *TSLP* genotype, the findings of Gauvreau et al.[33] and a follow-up larger phase 2b study of asthma suggest that anti-TSLP therapy may be beneficial to *select* individuals with poorly controlled asthma[34].

Our data illustrate the key role of *TSLP* genotype in the modulation of asthma disease expression and its management, and the influence of TSLP genetic variation on disease expression/endotype; this is aligned with previous observations on the targeted use of biologics, such as anti-IL5/IL5R therapies, in eosinophilic asthma[35,36], and the concept of specialized risk-stratification and treatment of asthma endotypes.

In summary, our novel data suggest that the rs1837253 T allele may produce asthma protective effects through differential binding and regulating the expression of lftTSLP, ultimately mitigating allergic inflammatory responses. Further investigation into mechanistic pathways regulated by the T allele, to produce an asthma-mitigating effect, is warranted. This information will allow us to more fully understand the role of TSLP in allergic asthma.

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DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon request.

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Tables:

TABLE 1: CHARACTERISTICS OF THE STUDY POPULATION

Characteristics	Asthmatic	Non-Asthmatic
Sample Size, N	15	30
Sex, male:female	7:8	16:14
Age, years (mean)	39	29.2
Atopy, n	14	23
Ethnicity		
Asian	2	7
Caucasian	10	13
Other	3	10
Genotype		
Homozygous major (CC)	7	12
Heterozygous (CT)	7	11
Homozygous minor (TT)	1	7

FIGURE LEGENDS

Figure 1: Relationship between asthmatic status and poly(I:C)-induced secretion of (A) TSLP (B) IL-5 (C) GM-CSF (D) INF- γ (E) IL-8 (F) TNF- α in nasal epithelial cells. Concentrations of secreted cytokine and chemokine protein in nasal epithelial cell culture supernatant from asthmatic (n=13) and non-asthmatic (n=25) individuals were measured using Bio-Rad Bio-Plex assays following 24h stimulation with 25 μ g/mL of poly(I:C). One independent experiment was performed per subject. Statistical testing was conducted using students t-tests. (*p<0.05).

Figure 2: Relationship between TSLP rs1837253 genotype and poly(I:C)-induced secretion of (A) TSLP (B) IL-5 (C) GM-CSF (D) INF- γ (E) IL-8 (F) TNF- α in nasal epithelial cells. Concentrations of secreted cytokine and chemokine protein in nasal epithelial cell culture supernatants from rs1837253 homozygous major allele (CC; n=17), heterozygous (CT; n=14), and homozygous minor allele (TT; n=7) individuals were measured using Bio-Rad Bio-Plex assays following 24h stimulation with 25 μ g/mL of poly(I:C). One independent experiment was performed per subject. Statistical testing was conducted using one-way ANOVA with Tukey post-hoc analysis. (*p<0.05)

Figure 3: Poly(I:C) induces TSLP gene expression in human primary nasal epithelial cells (NECs). Gene expression kinetics of the (A) total TSLP (B) long TSLP isoform (C) short TSLP isoform (n = 40). Results shown are expressed as fold change in the mRNA level in poly(I:C)-stimulated NECs at 3h and 6h relative to the level in unstimulated NECs (0 h). Statistical testing was conducted using two-way ANOVA with Bonferroni post-hoc analysis. (*p<0.05, **p<0.01, ***p<0.001)

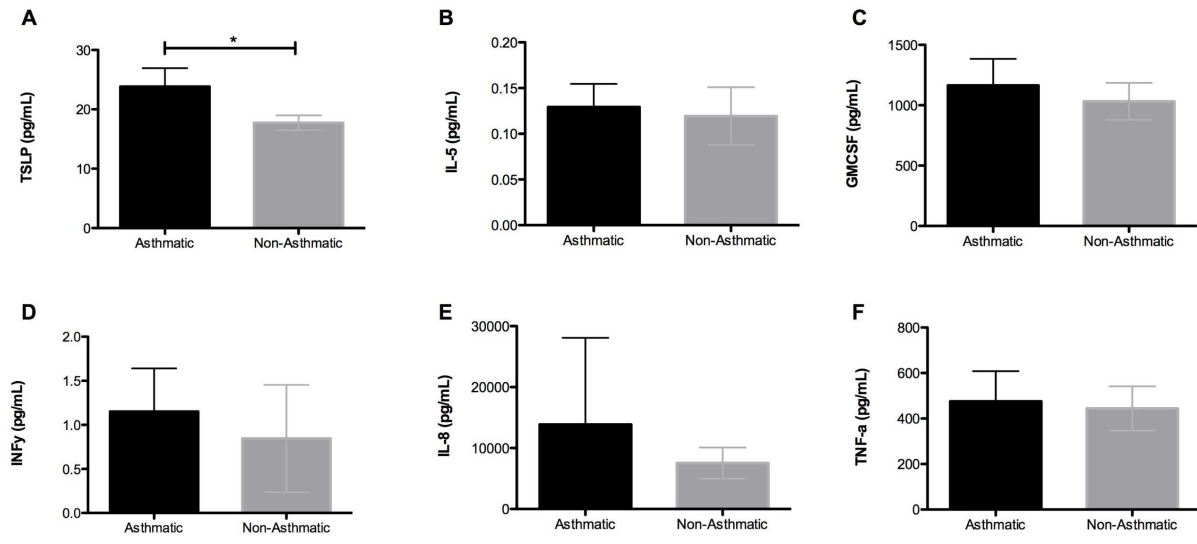
Figure 4: Poly(I:C) induces the expression of the long isoform of TSLP differently according to genotype of SNP rs1837253. Gene expression kinetics of the (A) total TSLP (B) long TSLP isoform (C) short TSLP isoform (n = 40). Results shown are expressed as fold change in the mRNA level in poly(I:C)-stimulated NECs at 3h and 6h relative to the level in unstimulated NECs (0 h) and are stratified by genotype. Statistical testing was conducted using two-way ANOVA with Bonferroni post-hoc analysis. (* indicates significant difference between homozygous major and heterozygous allele groups, ** indicates significant difference between homozygous major and homozygous minor allele groups; *, ** indicates p<0.05).

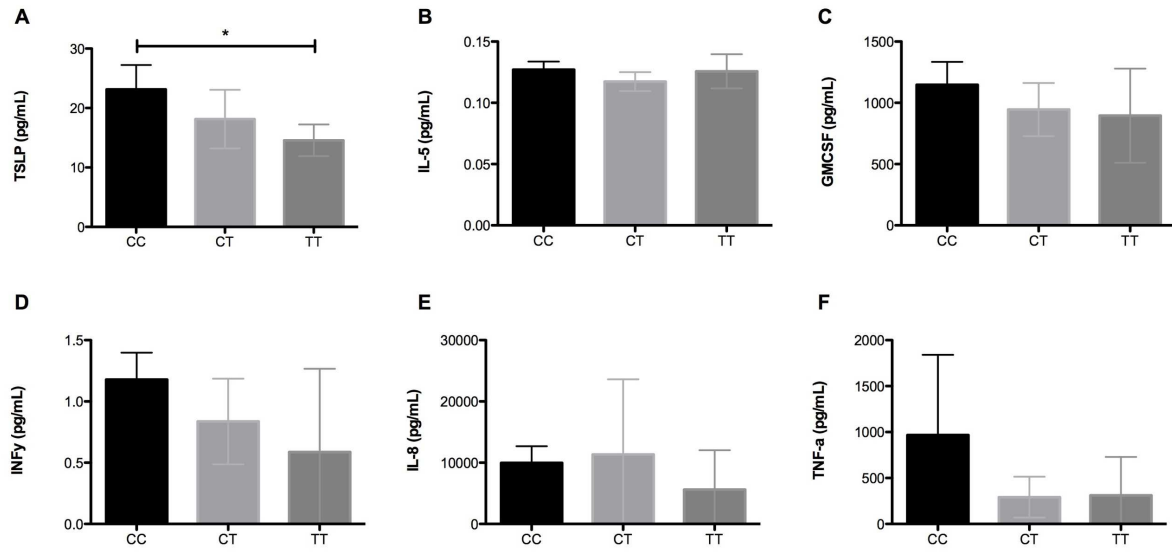
Figure 5: Relationship between genotype and asthmatic status in poly(I:C)-induced secretion of TSLP.

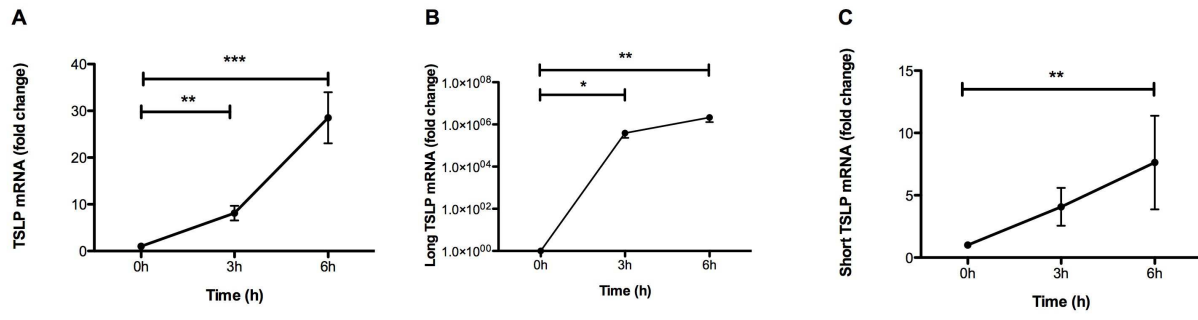
Gene expression analysis comparing the (A) total TSLP (B) long TSLP isoform (C) short TSLP isoform, given the presence (T) or absence (C) of the minor T allele in asthmatic subjects compared to non-asthmatic subjects at 6 h. Statistical testing was conducted using two-way ANOVA with Bonferroni post-hoc analysis. (* $p < 0.05$).

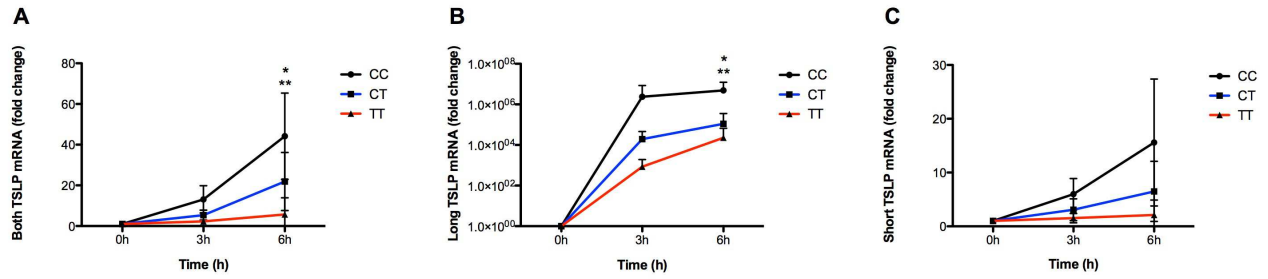
Figure 6: Luciferase ratio difference between rs1837253 alleles (N=34 in duplicate in 6 independent experiments)

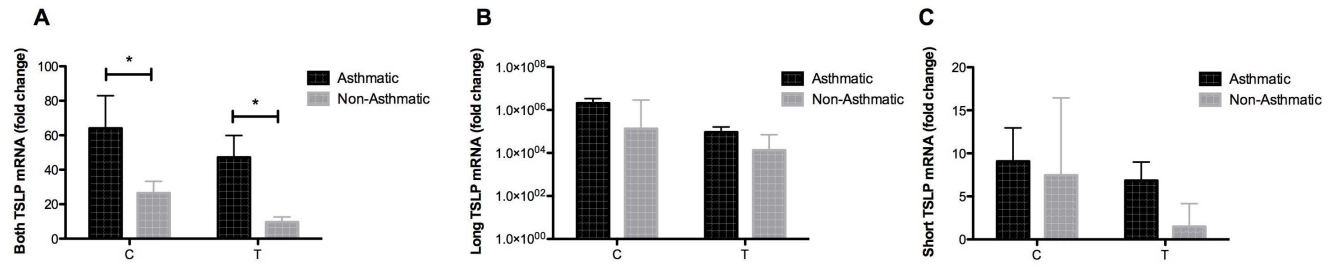
Figure 7: *Effect of poly(I:C) treatment on Luciferase activity of A549.* The x axis represents the treatments by allele: C and T are the rs1837253 allele-specific plasmids; C indicates cells transfected with the C allele, T indicates cells transfected with the T allele; C0 and T0: no treatment; C10 and T10: poly(I:C) treatment at 10 $\mu\text{g/ml}$; C20 and T20: poly (I:C) treatment at 20 $\mu\text{g/ml}$, C30 and T30: poly (I:C) treatment at 30 $\mu\text{g/ml}$.

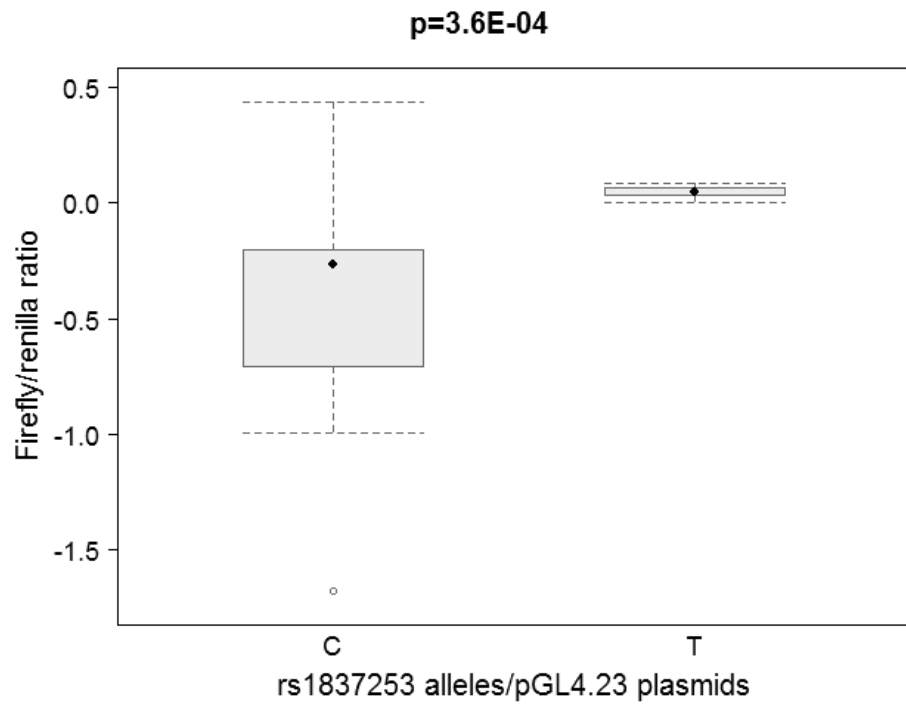


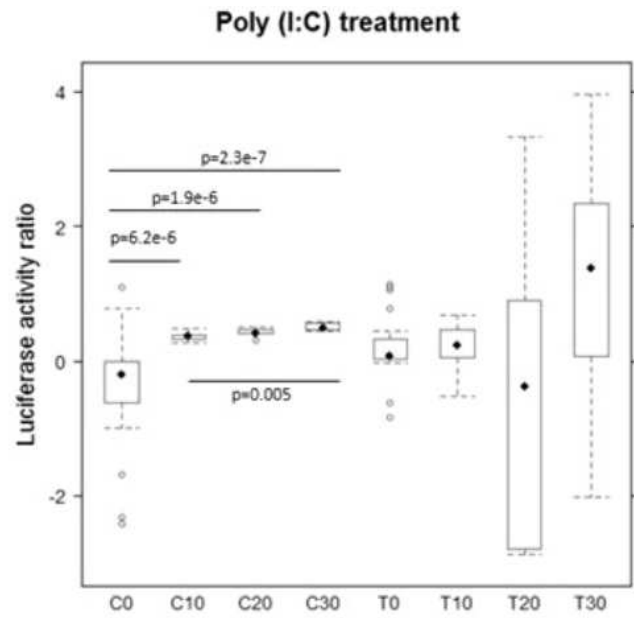












A thymic stromal lymphopoietin polymorphism may provide protection from asthma by altering gene expression

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SUPPLEMENTARY INFORMATION

METHODS

NASAL EPITHELIAL CELL ISOLATION, CULTURE AND POLY(I:C) STIMULATION

Primary nasal epithelial cells (NECs) were derived from the inferior nasal turbinate using Rhino-Probe nasal curettes (Arlington Scientific, Springville, UT). NECs were expanded and cultured as previously described by Muller *et al.*[25] with some modification. NECs were maintained in bronchial epithelial growth medium (Clonetics BEGM™; Lonza, Walkersville, MD) supplemented with Lonza SingleQuots™ kits. The SingleQuots™ kits were comprised of 2 ml bovine pituitary extract (52mg/mL, 0.5 ml of insulin (0.5 µg/mL), 0.5 ml of human epidermal growth factor (0.5 µg/mL), 0.5 ml of hydrocortisone (0.5 µg/mL), 0.5 ml of epinephrine (0.5 µg/mL), 0.5 ml of transferrin (0.5 µg/mL), 0.5 ml of retinoic acid (0.5 µg/mL), 0.5 ml of triiodo-L-thyronine (0.5 µg/mL), and 0.5 ml of Gentamicin/Amphotericin-B (GA1000; (0.5 µg/mL).

Collected cells were centrifuged and resuspended in BEGM with 10% 100x DNase 1 (Sigma Aldrich, St Louis, MO) at room temperature for 20 minutes. Cells were washed twice and plated on 35 mm culture dishes (Corning Costar, Corning, NY) coated with Purecol (Inamed, Fremont, CA) and incubated at 37°C, 5% CO₂. Medium was added 24 hours after plating, after which it was replaced every second day until cells reach 80-90% confluency. Second and third passaged cells were used for analysis.

Poly(I:C) stimulation was performed as previously described by Hui *et al.*[20]. Briefly, NECs were seeded onto 100mm dishes (Corning) and allowed to grow to 80-90% confluence. BEGM was replaced with bronchial epithelial basal media (Lonza, Walkersville, MD) and 1% fetal bovine serum (R&D Systems, Minneapolis, MN) before experimental stimulation. NECs were treated with 25µg/mL poly(I:C) (EMD Chemicals, Gibbstown, NJ) as indicated or vehicle control for 3h, 6h, or 24h at 37°C, 5% CO₂.

Protein levels of TSLP, GM-CSF, IFN γ , TNF- α , IL-8 and IL-5 from nasal epithelial cell culture supernatants following 24h poly(I:C) stimulation, were assessed using Bio-Plex assays (Bio-Rad, Hercules, CA) according to manufacturer's recommendation. The detection limits for these cytokines were 3.1 pg/mL for TSLP, 9.5pg/mL for GM-CSF, 0.1 pg/mL for IFN γ , 0.1 pg/mL for TNF- α , 0.2 pg/mL for IL-8 and 0.1 pg/mL for IL-5.

RNA ISOLATION AND REVERSE TRANSCRIPTION

RNA was extracted from human nasal epithelial samples using RNeasy Mini-kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA for each sample was quantified using a NanoDrop Spectrophotometer (ThermoFisher, Wilmington, DE). The RNA from each time point (0h, 3h, and 6h) was diluted based on the lowest concentration sample using RNase-free water. The samples were reversed transcribed using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to manufacturer's instructions and complementary DNA samples were aliquoted and stored at -80°C.

QUANTITATIVE REVERSE TRANSCRIPTION-PCR

TSLP gene expression was determined using SsoAdvanced™ Universal® SYBR Green Supermix (Bio-Rad, Hercules, CA) with a MX4000 Stratagene detection system according to the manufacturer's instructions. The PrimePCR Human reference Gene Panel (Bio-Rad Hercules, CA) was used to determine glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and tyrosine 3 monooxygenase/tryptophan 5-mono-oxygenase activation protein (*YWHAZ*) to be the optimal reference genes. Primer sets were previously verified by Hui and colleagues[20] using 8-point standard curves to confirm adequate amplification efficiency for each *TSLP* isoform primer sets

and the *GAPDH*/*YWHAZ* reference genes using Sso Advanced™ SYBR® Green Supermix (Bio-Rad Hercules, CA).

The following primer set for quantitative reverse transcription-PCR was used: (NM_033035.4 and NM138551.4), 5'CTAAGGCTGCCTTAGCTATC-3' and 5'AAGCGACGCCACAATCCTTG-3'. Prime PCR SYBR Green assay for Human *GAPDH* and *YWHAZ* were used as reference gene primers (Bio-Rad Hercules, CA). All *TSLP* primer sets (Integrated DNA Technologies, Coralville, IA) were designed and evaluated as per the minimum information for publication of quantitative reverse transcription-PCR experiment (MIQE) guidelines. Pooled complementary DNA was used to optimize annealing temperature (found to be 60°C for all primer sets).

Melt curves were used to verify single peak amplicons. The Threshold cycle (Ct) is inversely correlated with the target mRNA expression level and was defined as the cycle number at which the reporter fluorescence emission exceeded the midpoint along the amplification curve. The standard $2^{(-\Delta\Delta Ct)}$ formula was used to calculate arbitrary *TSLP* mRNA concentration. The level of *TSLP* mRNA was normalized to the level of *GAPDH*/*YWHAZ* mRNA.

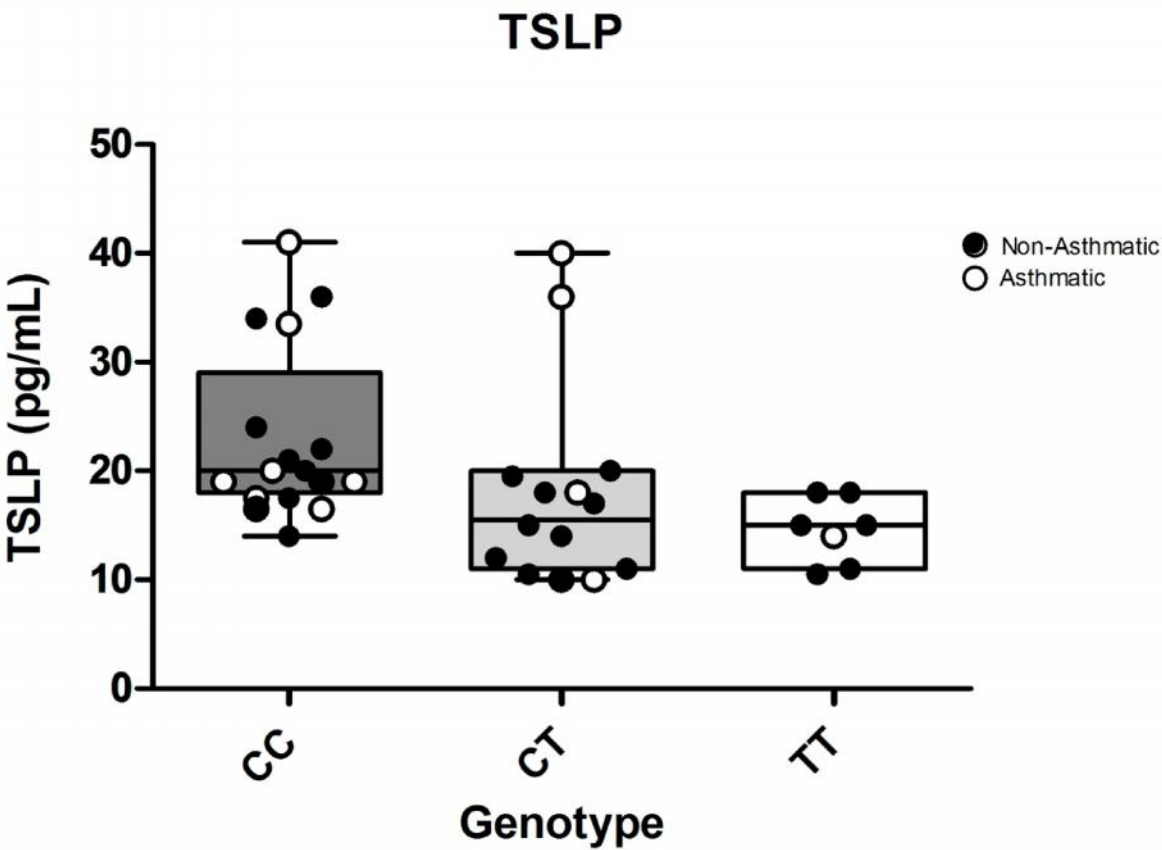
REPORTER GENE ASSAY

DNA inserts for the *TSLP* rs1837253 T allele were amplified using specific primers on a DNA from a homozygote sample. The DNA insert for the C allele was constructed using site-directed mutagenesis using the Stratagene Quickchange site-directed mutagenesis kit (Agilent technologies Inc., Santa Clara, CA). These DNA fragments were inserted into a pGL4.23[luc2/minP] vector using restriction enzyme sites. The vector used contains a 5' cloning site and a minimal promoter that drives luciferase expression. MAX Efficiency® DH5α™ Competent *E.coli* cells (Life Technologies) were used for transformation. pUC19 vector was used as a transformation control. The correct plasmid-carrying bacterial colonies were grown and used to extract DNA using Qiagen plasmid maxiprep kit (Qiagen) following the manufacturer's protocol. A549 cell cultures were transfected with 1 µg plasmid DNA for each SNP allele using Lipofectamine® LTX reagent for 24 hours. Co-transfection with Renilla plasmid DNA was performed as an internal transfection control at a 3 to 1 ratio (firefly to Renilla). Treatments were added 24-hour post-transfection and the cells were incubated for a subsequent 24 hours

83 then treated with poly(I:C) at 10, 20 and 30 $\mu\text{g/ml}$. Dual-Glo[®] Luciferase assay system
84 (Promega) reagents were used to measure Luciferase reporter gene activity for both firefly and
85 Renilla vectors. The ratio of luminescence from the experimental reporter (firefly) to the control
86 reporter (Renilla) was calculated after background-subtraction using control wells containing
87 empty vector that were treated using the same procedure.

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SUPPLEMENTARY FIGURE



SUPPLEMENTARY FIGURE LEGEND

Supplementary Figure 1: *TSLP protein expression from nasal epithelial cells stratified by rs1837253 genotype and asthmatic status.* Concentrations of secreted TSLP protein in nasal epithelial cell culture supernatants from individuals were measured using Bio-Rad Bio-Plex assays following 24h stimulation with 25µg/mL of poly(I:C). The box and whisker plot indicate the levels of TSLP expression for each genotype as labelled and the dot plot indicates the asthmatic status of the subjects. One independent experiment was performed per subject.

TSLP

